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IMMUNOLOGICAL STUDIES OF ANTHRAX
III. COMPARISON OF ANTIBODY TITER AND IMMUNITY INDEX FOLLOWING ANTHRAX IMMUNIZATION

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ABSTRACT

The effects of anthrax immunization on the serum antibody titer and the immunity index were compared for guinea pigs and rats immunized by two protocols. An association between the two measures could not be detected in either species of animals immunized at any one level; however, when data on animals immunized at several levels were combined a weak but significant association became apparent. The results of the agar diffusion test were more variable than those of the immunity index, and at relatively low or intermediate levels of protection the agar diffusion test produced a much larger portion of false negatives. This weakness did not carry through to animals protected to a high level of resistance, although at nearly all levels of protection the immunity index was more precise than the serological test. A comparison of three antigens administered by two protocols showed differences in both titer and index attributable to antigen. In this study only the Belton and Strange antigen significantly increased both the immunity index and the serum antibody titer.

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I. INTRODUCTION

Although evaluation of in vivo response to an antigen usually is determined by change in antibody titer, titer may be an imperfect measure of disease resistance. The agar-diffusion method of Thorne and Belton[1] to titrate antigen or antibody recently was used by Norman et al[2] in an attempt to detect subclinical anthrax among workers of goat hair processing plants. Their data suggest the occurrence of undetected cases and also indicate that humans immunized with protective antigen show no lasting titer. In earlier papers,[3-4] we introduced and used the immunity index as a measure of immune response. This index represents the log increase in challenge dose required for the immunized host to give the same response as the non-immunized host. Because of the infrequency of occurrence of anthrax, it is probable that the relationship between antibody titer and resistance to infection in human populations will never be conclusively shown; however, populations of laboratory animals can be surveyed for the correlation between the indirect and direct observation and inferences drawn regarding human populations. This paper presents the results of such a comparison using populations of guinea pigs and rats. The immunity index proves to be a more sensitive, precise measure of immunity than antibody titer, and we observe that caution must be exercised in drawing immunity inferences based on titer alone.

II. MATERIALS AND METHODS

A. ANIMALS

Guinea pigs of the Hartley strain obtained from the Fort Detrick animal farm and black rats developed from Long-Evans stock obtained from the National Institutes of Health aniaml farm were used.

B. CHALLENGE ORGANISM

Spores of the Vollum strain were cleaned by washing 16 times in distilled water. Spores were tested for cleanliness by: (a) absence of methylene-blue-stainable material and (b) absence of catalase in the washed spores as evidenced by lack of oxygen produced from superoxal (20 per cent H_2O_2). The challenge dose of all animals was 1×10^9 cleaned spores.

C. PROTECTIVE ANTIGEN

Three preparations of protective antigen were used. The Belton and Strange[5] antigen was prepared by the method of Strange and Thorne,[6] and corresponds to their crude antigen. It gave only one line of precipitate in agar diffusion plates when tested against equine spore antiserum. All crude culture filtrates that were used titrated at 1:16. In serological tests antigen was used in 1:3 dilution with the test serum. For immunization studies, crude culture filtrates were concentrated and stabilized by alum precipitation[5] to an agar diffusion titer of 1:128. The Wright and Puziss[7] deep culture antigen was prepared from a culture of strain V770-NPI-R grown anaerobically and adsorbed on aluminum hydroxide gel. This crude antigen had a complement fixation titer of 1:160 and an agar diffusion titer of 1:16. The Puziss and Wright[8] shallow culture antigen was prepared from a culture of strain RI-NP. This antigen, grown aerobically, had a complement fixation titer of 1:80 and an agar diffusion titer of 1:4 before concentration by alum precipitation. When tested with the linear pattern, all antigens gave only one line of precipitate. Antigens were administered to either rats or guinea pigs for immunization as outlined below.

D. LIVE VACCINE

Spores of the 30R strain, a rough mutant selected from the Vlb strain of Bacillus anthracis, were cleaned by the same procedure as was used for challenge organisms. Vaccine was stored at 4°C in a concentration of 10^{10} spores/ml.

E. REFERENCE ANTISERUM

Equine hyperimmune serum (DH-1-4A) prepared by repeated injections with spores of the Sterne strain of B. anthracis was furnished by Dr. Curtis B. Thorne.

F. IMMUNIZATION PROCEDURES

Immunization procedures involving the intraperitoneal injection of protective antigen (PA-5), protective antigen plus live vaccine (PA-5 + LV), live vaccine alone (LV), and protective antigen plus a booster of protective antigen (PA-5 + PA) described by Klein et al[4] were used for this study on guinea pigs and rats.

In a separate study involving 432 guinea pigs immunized with several combinations and doses of antigens, we were unable to demonstrate any difference in level of immunity between animals immunized through the intraperitoneal route and animals immunized through the intramuscular route. We, therefore, continued to use the intraperitoneal route in these studies.

G. TEST SERA

Blood was collected from rats and guinea pigs by cardiac puncture one week following administration of the last antigen injection. Untreated control animals were bled in the same manner and on the same time sequence as the treated animals. Deaths due to cardiac puncture in the first study amounted to 4 per cent, i.e., 12 of the 300 guinea pigs and 9 of the 225 rats. These animals are not included in any of the subsequent totals. No deaths resulted from this procedure in the second study. Serum was separated from whole blood by centrifugation, frozen and stored at -20°C until titrated.

H. SEROLOGICAL TEST

Noble agar plates with three parallel rows of wells were prepared by the method of Thorne and Belton.[1] The middle wells were filled with undiluted reference antiserum and the plates were incubated for six hours at 30°C. Test serum-antigen mixtures consisting of 0.2 ml of protective antigen mixed in test tubes containing 0.2 ml of serial twofold dilutions of animal test serum were shaken by hand and added to the outer rows of wells in the agar plates. The plates were held at a constant temperature of 30°C for 48 hours and then read for lines of precipitation. Titers were recorded as the highest dilution of serum that prevented precipitation. For controls, hyperimmune antiserum and antigen were titrated with each assay of unknown test serum. Duplicate plates were made for each animal. Each titer reported here is the average of the two observations, although

the duplicate readings were different in only 10 of the 225 pairs of plates for rats and in 28 of 234 pairs of plates for guinea pigs. All these cases disagreed by a factor of one dilution only.

I. IMMUNITY INDEX

The levels of immunity attained were described in terms of the immunity index.[3] This index represents the difference in challenge dose (in logs) required to cause the same response in immunized and control animals. Since previous work showed that the dose-response curves of immunized and non-immunized animals are statistically parallel, the immunity index, I, was computed for each animal using the equation:

$$I = \frac{1}{b} (X_1 - X_2) \qquad (1)$$

where b is the slope (1) of the dose-response curve for the species of animals being considered, X_1 is the mean reciprocal response time of controls, and X_2 is the reciprocal response time of the immunized animals for which the index is being computed. For any animal that survived, i.e., did not die of anthrax within 60 days of challenge, $X_2 = 0$. For these animals the index was found as:

$$I = \frac{X_1}{b} \qquad (2)$$

J. EXPERIMENTAL PROCEDURES

Experimental results reported in this article came from two independent studies. The first was designed to compare the antibody titer with the immunity index as measures of anthrax immunization. The second was designed to compare the antigenic activity of antigen produced from the Sterne strain with that of antigens from two other nonencapsulated strains of *B. anthracis*. In the first study two independent replications were performed using in total the following number of animals for each protocol tested:

Protocol	Rats	Guinea Pigs
PA-5	60	120
PA-5 + LV	60	120
LV	30	—
Controls	75	60
Total	225	300
Prechallenge Death	9	12
Net	216	288

In the second study one replicate was performed using the following number of guinea pigs for all antigens and protocols tested:

Protocol	ANTIGEN Deep Culture	Shallow Culture	Belton and Strange
PA-5	12	12	12
PA-5 + PA	12	12	12
Controls	12	12	12
Total	36	36	36

All animals provided two measures. The first, serum antibody titer, was measured in blood collected one week following immunization. One week after the blood was collected the animals were challenged. This provided data from which the second measure, immunity index, was computed. Two highly immune groups of rats did not die and, therefore, furnished us with no information from which we could examine the correlation between immunity index and serological test.

III. RESULTS

A. IMMUNITY INDEX AND ANTIBODY TITER OF GUINEA PIGS AND RATS

In Table I a distribution based on titer and immunity index is presented for 115 guinea pigs protected with PA-5 alone. The correlation coefficient between titer and immunity index computed from these data is r = 0.24, which is statistically significant at the five per cent level. The same procedure was followed for guinea pigs protected by PA-5 + LV. The bivariate classification of the 109 animals is shown in Table II. The correlation coefficient computed from these data is r = 0.06, which is not statistically significant.

TABLE I. DISTRIBUTION OF 115 GUINEA PIGS IMMUNIZED BY PA-5 BY SERUM TITER AND IMMUNITY INDEX

	Immunity Index						
Serum Titer	Less than 0.0	0.0 to 1.9	2.0 to 3.9	4.0 to 5.9	6.0 to 7.9	8.0 to 9.9	Total
<1/2	6	18	21	16	13	1	75
1/2 - 1/4	0	1	2	0	0	0	3
1/4 - 1/8	2	3	5	4	3	0	17
1/8 - 1/16	0	1	2	3	5	0	11
1/16 - 1/32	0	1	1	2	3	0	7
1/32 - 1/64	0	0	0	1	1	0	2
Total	8	24	31	26	25	1	115

r = 0.242

The data from all 224 guinea pigs are presented in Table III. It is shown here that in the combined group the association between the two measures of immunity is much greater than in either group individually. The correlation coefficient r = 0.37 is statistically significant at the one per cent level.

TABLE II. DISTRIBUTION OF 109 GUINEA PIGS IMMUNIZED BY PA-5 + LV BY SERUM TITER AND IMMUNITY INDEX

	Immunity Index							
Serum Titer	Less than 0.0	0.0 to 1.9	2.0 to 3.9	4.0 to 5.9	6.0 to 7.9	8.0 to 9.9	10.0 to 11.9	Total
<1/2	0	0	0	0	0	0	0	0
1/2 - 1/4	0	0	0	0	0	0	0	0
1/4 - 1/8	0	0	0	0	0	0	0	0
1/8 - 1/16	0	0	0	0	1	0	0	1
1/16 - 1/32	0	0	4	0	6	8	4	22
1/32 - 1/64	0	1	1	3	12	4	2	23
1/64 - 1/128	0	1	1	3	9	11	2	27
1/128 - 1/256	1	0	0	2	18	4	2	27
1/256 - 1/512	0	0	0	0	9	0	0	9
Total	1	2	6	8	55	27	10	109

r = -0.063

The joint distribution of titers and indexes for 60 rats immunized with PA-5 is shown in Table IV. The correlation coefficient between the two measures is r = 0.10. This is not statistically different from zero. Similar information is given for 30 animals immunized with PA-5 + LV in Table V. Here the correlation coefficient r = 0.003.

The distribution presented in Tables IV and V was combined to give the distribution in Table VI. From this it is seen that there is a definite association between the serum titer and the immunity index, r = 0.46. This is statistically significant at the one per cent level.

These data indicate that the immunity index is associated with the serum titer. It has been shown in both species that this association is definite when a sufficiently wide range of the measures is considered. The extent of the association, however, is a different matter. In guinea pigs r = 0.37. This can be interpreted[9] by noting that only 14 per cent of the information provided by either measure is associated with the other. For rats the correlation coefficient r = 0.46 means that 21 per cent of the information provided by either measure is associated with the other.

TABLE III. DISTRIBUTION OF 224 GUINEA PIGS IMMUNIZED BY EITHER PA-5 OR PA-5 + LV BY SERUM TITER AND IMMUNITY INDEX

	Immunity Index							
Serum Titer	Less than 0.0	0.0 to 1.9	2.0 to 3.9	4.0 to 5.9	6.0 to 7.9	8.0 to 9.9	10.0 to 11.9	Total
<1/2	6	18	21	16	13	1	0	75
1/2 - 1/4	0	1	2	0	0	0	0	3
1/4 - 1/8	2	3	5	4	3	0	0	17
1/8 - 1/16	0	1	2	3	6	0	0	12
1/16 - 1/32	0	1	5	2	9	8	4	29
1/32 - 1/64	0	1	1	4	13	4	2	25
1/64 - 1/128	0	1	1	3	9	11	2	27
1/128 - 1/256	1	0	0	2	18	4	2	27
1/256 - 1/512	0	0	0	0	9	0	0	9
Total	9	26	37	34	80	28	10	224

r = 0.366

TABLE IV. DISTRIBUTION OF 60 NIH RATS IMMUNIZED BY PA-5 BY SERUM TITER AND IMMUNITY INDEX

	Immunity Index				
Serum Titer	Less than 0	0.0 to 1.9	2.0 to 3.9	4.0 to 5.9	Total
<1/2	6	24	10	1	41
1/2 - 1/4	1	13	4	0	18
1/4 - 1/8	0	0	1	0	1
Total	7	37	15	1	60

r = .102

TABLE V. DISTRIBUTION OF 30 NIH RATS IMMUNIZED BY PA-5 + LV BY SERUM TITER AND IMMUNITY INDEX

	Immunity Index				
Serum Titer	Less than 0	0.0 to 1.9	2.0 to 3.9	4.0 to 5.9	Total
<1/2	0	0	0	0	0
1/2 - 1/4	0	0	0	0	0
1/4 - 1/8	0	0	0	0	0
1/8 - 1/16	0	2	6	1	9
1/16 - 1/32	0	1	5	4	10
1/32 - 1/64	1	0	8	2	11
Total	1	3	19	7	30

r = .003

TABLE VI. DISTRIBUTION OF 90 NIH RATS IMMUNIZED BY EITHER PA-5 OR PA-5 + LV BY SERUM TITER AND IMMUNITY INDEX

	Immunity Index				
Serum Titer	Less than 0	0.0 to 1.9	2.0 to 3.9	4.0 to 5.9	Total
<1/2	6	24	10	1	41
1/2 - 1/4	1	13	4	0	18
1/4 - 1/8	0	0	1	0	1
1/8 - 1/16	0	2	6	1	9
1/16 - 1/32	0	1	5	4	10
1/32 - 1/64	1	0	8	2	11
Total	8	40	34	8	90

r = .458

Two groups of animals failed to provide both index and titer data, and therefore are not included in Tables IV or V. None of the 30 rats immunized with PA-5 + LV in the first replication died from anthrax. No definite index could be computed for them except that it must be greater than 3.01. It should be noted, however, that those animals that did not die, i.e., apparently had complete protection against a dose of 10^9 anthrax spores, had a high average titer of 1:104. None of the 30 animals immunized with live vaccine alone produced sufficient antibodies to be measured by immunodiffusion and they had a low average immunity index of 2.3. These results again indicate that a low index is associated with a low titer, which in turn is generally associated with poor protection against anthrax. They also indicate that a high index indicates good protection and may or may not be accompanied by a high titer.

B. CULTURE (STRAIN) ANTIGENICITY

In our second study, Table VII, designed to compare the antigenic activity of three antigens administered by two protocols, PA-5 and PA-5 + PA, we again demonstrated that, within the relatively narrow range of immunity obtained, there was no correlation between antibody titer and immunity index. As in the first study, however, we found for two antigens a statistically significant although very weak correlation between the two measures when animals immunized at two levels were considered. Even with the wider range of immunities, the correlation between the two measures was not significant when the deep culture antigen was used.

Although these two measures are probably indicative of the antigenicity of the three cultures we find that they are not in complete agreement. Thus, as shown in Table VIII, the deep culture antigen when used as a booster increases the immunity as measured by the titer. There is, however, no corresponding increase in the index. Conversly shallow culture antigen, when used as a booster significantly increases immunity as measured by the immunity index but not by the titer. In contrast to both of these, a booster of the Belton and Strange antigen significantly increased both the immunity index and the serum antibody titer.

TABLE VII. CORRELATION BETWEEN ANTIBODY TITER AND IMMUNITY INDEX AMONG GUINEA PIGS IMMUNIZED WITH THREE ANTIGENS BY TWO PROTOCOLS

Culture	Immunization Procedure	Number Animals	Correlation Coeff.
Deep	PA-5	12	0.09
	PA-5 + PA	12	0.08
	Both	24	0.12
Shallow	PA-5	12	0.24
	PA-5 + PA	12	0.02
	Both	24	0.55*
Belton & Strange	PA-5	12	0.56
	PA-5 + PA	12	0.10
	Both	24	0.53*

* Statistically significant at the five per cent level.

TABLE VIII. MEASURES OF IMMUNITY FOR GUINEA PIGS IMMUNIZED AT TWO LEVELS WITH THREE ANTIGENS

Measures	Deep Cultures		Shallow Cultures		Belton-Strange	
	PA-5	PA-5 + PA	PA-5	PA-5 + PA	PA-5	PA-5 + PA
Mean Index	3.3	3.8	1.3	5.2	2.4	4.8
S.E. of Mean Index	.41	.47	1.4	.70	.25	.52
CV of Index, %	12.4	12.4	107.0	13.5	10.4	10.8
Proportion Responding with Titer	5/12 (42%)	12/12 (100%)	2/12 (17%)	6/12 (50%)	3/12 (25%)	12/12 (100%)
Range of Titer	<1/2-1/8	1/4-1/12	1/2-1/4	<1/2-1/16	<1/2-1/8	1/8-1/128
Mean Titer	1/1.5	1/7.6	<1/2	1/4	1/1.3	1/45
S.E. of Mean Titer	1/.44	1/.43	1/.13	1/3.55	1/.57	1/173.6
CV of Titer, %	29.3	5.7	6.5	88.8	43.8	385.8

IV. DISCUSSION

The principal purpose of this paper is to compare two methods of measuring protection against challenge with *B. anthracis*. The agar diffusion method suggested by Thorne and Belton[1] does not require the sacrifice of the animal, whereas the immunity index suggested by DeArmon et al[3] does. We have found that both methods are capable of detecting gross differences in the level of immunity; however, where a sacrifice may be made the immunity index is both a more sensitive and more precise measure of immunity. The fact that we could measure an immunity index on 69 of the 75 guinea pigs (Table III, row 1) and on 35 of the 41 rats (Table VI, row 1) that showed no measurable titer is ample evidence that the index is a more sensitive measure of immunity than the serological test.

The mean level and precision of each measure in each species immunized by each protocol is shown in Table IX. Coefficients of variation (CV) are computed as 100 times the ratio of the standard deviation to the mean of each measure. A high CV indicates that there is a large amount of variation relative to the mean and, hence, little precision. In the first study it is seen that protocol PA-5 + LV produces animals with a significantly higher level of immunity than protocol PA-5, whether this immunity is measured by the immunity index or by the serological test. For both species the mean index of PA-5 + LV animals is significantly higher than the mean index of PA-5 animals. This is true also of average titers. It is also notable that only about one-third of the PA-5-protected animals produced enough antibody to indicate a positive test, whereas all the PA-5 + LV animals were serologically positive. In the second study, summarized in Table VIII, it is seen that a booster of deep culture antigen has a marked effect on the agar diffusion titer and a booster of the shallow culture antigen influences the immunity index. On the other hand, a booster of the Belton and Strange culture antigen has a significant effect on both titer and index. The inconsistency is not unexpected, since both the antiserum and the antigen used were developed from the Sterne strain and may, therefore, react incompletely with test serum developed from other strains. Before any positive information can be developed, all combinations of antiserum and antigen must be tested. It may be noted also from Table VIII that in this study, as well as the first, the CV associated with the immunity index is generally smaller than the CV associated with the agar diffusion titer.

The wide range of titers associated with animals immunized by any given protocol should be considered carefully in the interpretation of this measure. One should also be well aware of the large proportion of false negatives that are likely to be observed among animals with a low to intermediate level of protection. We have shown, however, that when the immunity is great enough, as in animals protected with protocol PA-5 + LV, no false negatives occur when the serological test is used as a measure. It is still true, however, that even at the high level of immunity produced by PA-5 +LV the immunity index is a more precise measure than the agar diffusion titer and should be used whenever it is feasible to use death as an endpoint.

TABLE IX. MEASURES OF IMMUNITY FOR ANIMALS IMMUNIZED AT TWO LEVELS

Measures	Guinea Pigs PA-5	Guinea Pigs PA-5 + LV	Rats PA-5	Rats PA-5 + LV
Mean Index	4.4	7.1	1.0	3.2*
S.E. of Mean Index	3.25	1.98	.90	1.09
CV of Index, %	73.6	27.9	90.0	34.1
Proportion Responding with Titer	40/115 (34.7%)	109/109 (100%)	19/60 (31.7%)	30/30 (100%)
Range of Titer	1/2-1/64	1/8-1/512	1/2-1/6	1/16-1/64
Mean Titer	1/3.2	1/86.2	1/1.3	1/38.4
S.E. of Mean Titer	1/6.11	1/76.39	1/1.88	1/20.73
CV of Titer, %	190.9	88.6	144.6	54.0

* First replication of animals immunized with PA-5 + LV and animals immunized with LV were not included in calculating mean indexes or mean titers.

Although the work of Strange and Thorne[6] ". . . . provides a valid basis for the Thorne and Belton method of titrating B. anthracis immunizing antigen," we call attention to a partial failure under the conditions of our tests for its validity as a measure of immunity.

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